Biochimica et Biophysica Acta, 523 (1978) 516—521

BBA 68408

SELECTIVE PRECIPITATION OF $^{32}P_i$ ONTO FILTER PAPERS

APPLICATION TO ATPase AND CYCLIC AMP PHOSPHODIESTERASE DETERMINATION

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(Received August 31st, 1977)

Summary

A simple and selective method is described for the isolation of $^{32}P_i$ based on the precipitation of the phosphomolybdate complex with triethylamine. Precipitation takes place on filter papers, which are then washed with a solution containing ammonium molybdate and triethylamine to remove other radioactive phosphates. Very large numbers of samples and very small sample volumes can be accommodated easily. The use of this method to measure ATPase and cyclic AMP phosphodiesterase activity is demonstrated.

Introduction

The release of P_i is frequently used to monitor biochemical reactions. Colorimetric determination of P_i can be utilized if sufficient P_i is released. However, to increase sensitivity, it is sometimes necessary to measure the release of $^{32}P_i$ from ^{32}P-labelled substrates.

A number of methods have been described which are applicable to the isolation of $^{32}P_i$ from other ^{32}P-labelled compounds. Some of these methods utilize solvent extractions [1], column chromatography [2], adsorption of ^{32}P-labelled nucleotides onto charcoal [3] and precipitation of the phosphomolybdate complex with treithylamine [4]. These methods can be quite tedious if large numbers of samples are involved. It occurred to us that the method of Sugino and Miyoshi [4] might be greatly simplified if one could wash the precipitates by some method other than centrifugation, e.g. by isolating the P_i precipitate on filter papers. Filter papers have been used in a variety of methods which

The abbreviations used are MES, 2-(*N*-morpholino)ethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

measure the transfer of radioactive substances from a soluble substrate to a precipitable substrate. These methods include measurements of the incorporation of nucleotides into DNA [5], glucosyl residues from UDP-glucose to glycogen [6] and phosphate groups from ATP into protein [7]. We report here that P_i can be quantitatively and selectively precipitated onto filter papers in the presence of molybdate and triethylamine. This allows one to easily measure, in a large number of samples, the release of $^{32}P_i$ from a ^{32}P-labelled substrate, e.g. [γ-^{32}P]ATP.

Materials and Methods

Materials. [γ-^{32}P]ATP, $^{32}P_i$, and cyclic[^{32}P]AMP were purchased from New England Nuclear, Boston, MA, and cyclic[8-^{3}H]AMP from Schwarz-Mann, Orangeburg, N.Y. All chemicals were reagent grade. Rabbit gastric mucosa was homogenized in 10 vols. 0.25 M sucrose and the homogenate was centrifuged at 10 000 $\times g$ for 15 min. The supernatant constituted 20% of the total reaction mixture in those experiments where it was utilized.

Reagents. (Prepared just prior to use.) A. Precipitating solution: 5.7% trichloroacetic acid, 2.9% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 29 mM triethylamine · HCl. B. Washing solution: 1.5% trichloroacetic acid, 0.74% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 7.4 mM triethylamine · HCl.

Isolation of $^{32}P_i$ on filter papers. 20 μl of solution containing $^{32}P_i$, e.g. an enzyme reaction mixture, were spotted onto filter papers (Whatman 31 ET, 1.5 × 2 cm). 15—30 s later the papers were dropped into a beaker containing 5 ml solution B/added paper. At 10-min intervals, the wash solution was replaced four times to remove other ^{32}P-containing compounds. The wash solutions were at room temperature. The papers were then oven dried on a glass plate, placed in vials containing 5 ml of toluene-based scintillation solution, and counted in a liquid scintillation counter.

Results

Effect of P_i concentration. In order to determine the optimal conditions for precipitation of P_i and to investigate effects of certain factors on P_i recovery, a simulated reaction mixture containing $^{32}P_i$ was used. The reaction mixture contained 50 mM MES (pH 6.5), $^{32}P_i$ (approx. 250 000 cpm/ml), 0.001—100 mM potassium phosphate (as carrier) and gastric mucosal supernatant (when included). Table I shows that recovery was greater than 90% when the P_i concentration was 0.1—10 mM. Increasing the P_i concentration to 30 mM or above markedly decreased recovery and decreasing P_i to below 0.1 mM slightly decreased recovery.

Effect of treating papers with solution A. The phosphate · molybdate · triethylamine complex does not precipitate instantaneously. Therefore it was considered useful to allow precipitation to occur on the papers prior to placing the papers in the washing solution. To accomplish this 25-μl aliquots of solution A were spotted on the filter papers 5—30 min prior to application of the $^{32}P_i$ sample. Table I shows that the use of papers treated with solution A increased the recovery of $^{32}P_i$ slightly (1—12%). The effect was greatest at the lowest and

TABLE I

EFFECT OF TREATING FILTER PAPERS WITH SOLUTION A AND EFFECT OF P_i CONCENTRATION ON THE RECOVERY OF P_i

Reaction mixtures of varying P_i concentration were prepared as described in the text. 20-μl aliquots were applied either to untreated filter papers or to filter papers that had been spotted with 25 μl of solution A. The papers were then washed and ^{32}P was determined as described in the text. Recovery is expressed as percent of total cpm applied to the filter paper.

P_i concentration (mM)	cpm ^{32}P recovered (%)	
	Untreated	Treated
100	33	37
30	61	73
10	92	95
1	91	95
0.3	92	94
0.1	91	93
0.03	86	88
0.01	86	87
0.003	83	89
0.001	82	86

highest concentrations of P_i. In the range of P_i concentration from 0.1 to 10 mM, recovery averaged 94.3% when the papers were treated with solution A and 91.5% when the papers were not treated. The recovery under both conditions was better than that obtained with isobutanol extraction (87%). Therefore the use of solution A is not necessary in this range of P_i concentration. However, to maximize recovery, treated papers were used in subsequent experiments.

Effect of volume of reaction mixture. Recovery of P_i was unaffected when the volume of reaction mixture applied to the papers was varied from 5 to 50 μl, provided that the amount of P_i applied did not exceed 0.3 μmol.

Effect of pH and of protein. In order to establish whether or not recovery of P_i was affected by pH of the reaction mixture, the 50 mM MES buffer was replaced with one of the following buffers: 50 mM acetate (pH 4), 50 mM acetate (pH 5), 50 mM TES (pH 7), 50 mM Tris (pH 8.5), or 50 mM glycine (pH 10). Each of these buffers was tested with or without the addition of the supernatant from gastric mucosa. In the absence of the supernatant recovery ranged from 95% at pH 4 to 102% at pH 8.5. Addition of the extract had no effect on recovery (range = 94—97%).

Applicability of the filter paper method for enzyme assays. It was important to establish that this method could be used for the measurement of enzyme activity, e.g. ATPase (EC 3.6.1.3) activity. Hydrolysis of 10 μM (Fig. 1A) and 2 mM [γ-^{32}P]ATP (Fig. 1B) to $^{32}P_i$ by an ATPase preparation from guinea pig kidney [8] was measured by the filter paper method and by the method of Siegel and Albers [9]. The latter method measures formation of $^{32}P_i$ by extraction of the phosphomolybdate complex into isobutanol. There was excellent agreement between the two methods. Hydrolysis of 10 mM [γ-^{32}P]ATP to $^{32}P_i$ by a supernatant of rabbit gastric mucosa was measured by the filter paper method and colorimetrically [10]. Again there was excellent agreement demon-

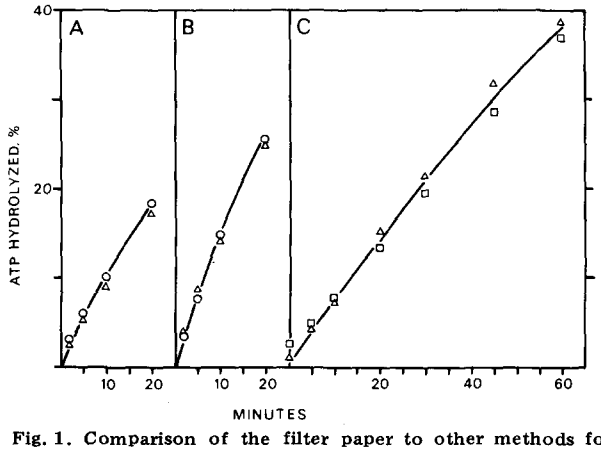

Fig. 1. Comparison of the filter paper to other methods for ATPase assay. In A and B the reaction mixture contained 100 mM Tris at pH 7.5, 60 mM NaCl, 25 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1% bovine serum albumin and ATPase from guinea pig kidney (8) at 30°. In addition, the reaction mixtures contained 10 μM ATP (230 cpm/pmol) in A and 2 mM ATP (1.3 cpm/pmol) in B. At the indicated times aliquots were removed for determination of $^{32}P_i$ by the filter paper method (△) and by the method of Siegel and Albers [9] (○). In C, the reaction mixture contained 50 mM TES at pH 7, 10 mM [γ-^{32}P]ATP (120 cpm/nmol), 20 mM magnesium acetate, 0.1 mM potassium phosphate, and supernatant of gastric mucosa. At various times, aliquots were removed for determination of $^{32}P_i$ by the filter paper method (△) and for colorimetric determination of P_i [10] (□).

strating the utility of this method over a 1000-fold range of ATP concentration and in the presence of high concentrations of protein. Proteins were precipitated by trichloroacetic acid prior to P_i determination so that the large amounts of protein present in this reaction mixture would not cause a precipitate when the colorimetric method or isobutanol extraction was used, but this step was not necessary when the filter paper method was used. In the

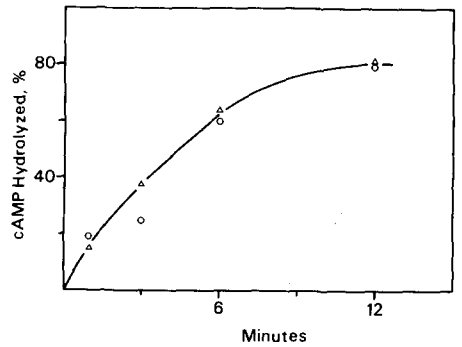

Fig. 2. Use of the filter paper method for determination of cyclic AMP phosphodiesterase activity. The reaction mixture contained 1.5 μM cyclic AMP, 100 mM Tris · Cl, 6 mM P_i, 8 mM $MgCl_2$, 0.8 mM EDTA, 0.02 mM dithiothreitol and an extract of rabbit stomach mucosa at pH 8.0 in a total volume of 1 ml. The reaction mixture also contained 30 nCi [^{32}P]cyclic AMP and 130 nCi [8-^{3}H]cyclic AMP. At the indicated times, 200 μl were removed, placed in a boiling water bath for 1 min and then placed on ice. 50 μl of a solution containing 50 μg *Crotalus atrox* venom and 50 nmol of adenosine were added to the aliquots. This mixture was incubated at 30°C for 10 min and placed on ice. $^{32}P_i$ (△) in 25 μl of these aliquots was determined as described above. [^{3}H]Adenosine (○) in 50 μl of these aliquots was determined essentially as described by Huang and Kemp [12].

absence of enzyme, approx. 2% of the radioactivity was recovered as $^{32}P_i$ with either method of measuring $^{32}P_i$ release. This is presumably due to $^{32}P_i$ in the [γ-^{32}P]ATP and indicates that under the conditions described there is minimal (<2%) non-enzymatic hydrolysis of ATP. This method has also been used successfully to measure ATPase activity in homogenous preparations of the catalytic subunit of the cyclic AMP-dependent protein kinase [11].

The application of this method to the determination of cyclic AMP phosphodiesterase (EC 3.1.4.17) is illustrated in Fig. 2. This enzyme catalyzes the hydrolysis of cyclic AMP to AMP. The activity of this enzyme can be conveniently measured by coupling the reaction to nucleotidase which converts AMP to adenosine and P_i. Release of [8-^{3}H]adenosine from cyclic[8-^{3}H]AMP was determined by a modification of the method of Huang and Kemp [12]. Release of $^{32}P_i$ from cyclic [^{32}P]AMP was measured by the filter paper method. As shown in Fig. 2, there was excellent agreement between the two methods.

Discussion

The method of precipitating P_i onto filter papers is adaptable to a variety of reaction conditions, since over 90% of the added $^{32}P_i$ could be recovered with a wide range of protein, phosphate, and hydrogen ion concentrations. The method is apparently specific for P_i since a variety of compounds including ATP (Fig. 1), cyclic AMP (Fig. 2), PP_i [4], dTTP [4] and glucose-1-*P* [4] are not precipitated. However, highly acid-labile phosphates can be hydrolyzed to P_i due to the acidic conditions needed for precipitation [4]. Acid-labile phosphates therefore would interfere with the determination of $^{32}P_i$ if they were radioactively labelled. The only interfering substances we have discovered are ^{32}P-labelled phosphoproteins, which can be precipitated by the conditions used to precipitate P_i. Therefore the method is not directly applicable in instances where high levels of ^{32}P-labelled proteins are present, e.g. protein phosphatase assays which utilize ^{32}P-labelled protein as the substrate. However, the interfering proteins can first be removed by precipitation with a protein precipitant. Agents which cause precipitation of the radioactive substrate would also interfere. Except for these limitations, this method appears to be applicable to the assay of a wide variety of enzymes which catalyze the release of P_i. In addition to ATPases and cyclic nucleotide phosphodiesterases, such enzymes include nucleotidases and phosphatases. The sensitivity of the method is limited primarily by the specific activity of the ^{32}P-labelled substrate and by the amount of $^{32}P_i$-contamination present in the substrate.

Because centrifugation and solvent extractions are eliminated, isolation of $^{32}P_i$ is much simplier than by previous methods. The method should also be advantageous when the reaction mixture contains amines which precipitate the phosphomolybdate complex. If $^{32}P_i$ is isolated by the solvent extraction method, it is necessary to remove such amines prior to addition of molybdate. The presence of such amines should not interfere when the complex is isolated by precipitation instead of by solvent extraction. In addition the method has an advantage over other methods in that ^{32}P remains on the filter papers when they are placed in vials for liquid scintillation counting. Therefore the liquid

scintillation solution can be used repeatedly because it does not become contaminanted with ^{32}P. This results in considerable savings of money and greatly simplifies the disposal of radioactive materials.

Acknowledgements

We thank Dr. George Henderson for the ATPase from guinea pig kidney and Dr. Keith Schlender for reviewing the manuscript. This research was supported by USPHS Grant AM 15611. We thank Cindy Licata for the typescript.

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